

**Application
for
United States Letters Patent**

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To all whom it may concern:

Be it known that KE-WEN DONG, SERGIO C. OEHNINGER AND
WILLIAM E. GIBBONS

has invented certain new and useful improvements in

HUMAN ZONA PELLUCIDA PROTEIN 3 AND USES THEREOF

of which the following is a full, clear and exact description.

5

HUMAN ZONA PELLUCIDA PROTEIN 3 AND USES THEREOF

This application claims priority of U.S. Serial No. 09/252,828, filed February 19, 1999, which claims
10 priority of U.S. Serial No. 60/075,079, filed February 19, 1998. The content of these applications is incorporated by reference into this application.

15 Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to
20 more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

A method to determine sperm activity and a
25 diagnostic kit for the same purpose.

BACKGROUND OF THE INVENTION

30 Earlier studies have led to the characterization of the protein components of the zona pellucida (ZP) from a variety of mammalian species, with the majority of the work being performed in the murine and porcine species. The reported number of
35 discrete proteins comprising the ZP from different mammalian species varies (Dunbar et al, 1981; 1994; Timmons and Dunbar, 1988; Bleil and Wassarman, 1980). Murine studies have revealed that the ZP is composed of three sulfated glycoproteins, referred
40 to as ZP1, ZP2 and ZP3. Extensive work in this model has resulted in the identification and isolation of the primary receptor for sperm located at the level

5 of the zona pellucida, a glycoprotein called zona
 pellucida protein 3 (ZP3) (Bleil and Wassarman,
 1980; Wassarman, 1990 a and b). The binding of sperm
 to ZP is supported by ZP3 and complementary binding
 10 protein(s) present in the sperm plasma membrane
 (Saling, 1989; Wassarman, 1990; Saling, 1991).
 Genes homologous to the ZP2 and/or ZP3 genes have
 been cloned for the mouse (ZP3 and ZP2), hamster
 (ZP3), human (ZP3), rabbit (rc75), and marmoset
 (Ringuette et al., 1986; Liang et al., 1990; Kinloch
 15 et al., 1990; Chamberlin & Dean, 1990; Liang & Dean,
 1993; Lee et al., 1993; Thillai-Koothan et al.,
 1993). Genes encoding ZP2 and ZP3 are conserved
 among mammals and sequences of ZP3 cDNA coding
 regions show extensive homology between species
 20 studied so far.

Cloning cDNAs encoding ZP3 has made the expression
 of recombinant ZP3 in tissue culture cell lines
 possible and represents the potential to obtain
 25 large amounts of recombinant ZP3. The expression of
 biologically active recombinant ZP3 has been
 reported, at least, in the mouse (Kinloch et al,
 1991; Beebe et al, 1992) and human (van Duin et al,
 1994; Barratt et al, 1994; Burks et al, 1995). In
 30 the mouse, some of these recombinant proteins have
 demonstrated partial or full biological activity in
 ligand-receptor or acrosome reaction assays.
 Expression of recombinant ZP proteins is not
 restricted to those of the mouse and human species.
 35 Prasad et al (1996) demonstrated that recombinant
 rabbit 55 kDa protein (which is thought to be the
 rabbit homologue of mouse ZP1) purified from a
 baculovirus expression system could be used to
 generate a polyclonal antiserum which was then

5 employed to study the localization of the native 55
kDa protein in rabbit zona.

On the other hand, recombinant human ZP3 has been
expressed using several approaches, i.e.,
10 *Escherichia coli* (Chapman and Barratt, 1996), in
vitro transcription and translation systems
(Whitmarsh et al, 1996), Chinese hamster ovary (CHO)
cells (van Duin et al, 1994; Barratt and Hornby,
1995; Brewis et al, 1996) and in African green
15 monkey kidney (COS) cells (Burks et al, 1995). In
the human, however, full biological activity, which
includes the ability to bind spermatozoa in a
specific fashion and to induce the acrosome
reaction, has not been fully demonstrated for such
20 products. This is possibly due, among other reasons,
to inadequate or incomplete glycosylation of the
recombinant protein (Chapman and Barratt, 1997).

In the human system, production of a pure
25 recombinant ZP3 glycoprotein in a biologically
active form has been fraught with technical
difficulties. Expressing recombinant ZP3 protein
with *in vitro* transcription and translation systems
and in *Escherichia coli* has shown a variable
30 acrosome reaction-inducing activity. However, no
direct or specific sperm-binding ability using
homologous sperm-ZP bioassays has been reported for
such non-glycosylated products. In addition, protein
solubility has been a major difficulty encountered
35 (Chapman and Barratt, 1997). The rhZP3 expressed in
CHO cells has been shown to possess acrosome
reaction-inducing activity. However, no data are
available regarding sperm binding in validated
assays (van Duin et al, 1994; Barratt and Hornby,

5 1995). The fact that such recombinant proteins lack full sperm binding activity points to inadequate glycosylation of the protein core by the host cells.

10 In our studies we have cloned and expressed the cDNA of human ZP3 by stable transfection in a human ovarian cell line (PA-1 cells). This cell line was chosen to fit the glycosylation criterion, since glycosylation is tissue- and species-specific (Varki, 1993). The PA-1 cells produce glycosylated
15 proteins such as lactosaminoglycan-carrier glycoprotein (Fukuda et al, 1985), heparin-binding protein (Furukawa et al, 1990) and fibronectin (McIlhinney and Patel, 1983), and have been successfully used as an expression host to express
20 other glycoproteins such as Interleukin-6 receptor (Lust et al, 1995). We purified the recombinant glycoprotein product and characterized its biological activities as sperm ligand (in competition studies using a homologous sperm-zona
25 pellucida binding bioassay) and as physiologic inducer of the acrosome reaction (triggering exocytosis of sperm in suspension and assessing the frequency of acrosome reaction by lectin binding fluorescence). A first description of the full
30 biological activities of this product has been reported (Dong et al, 2000). Here we have focused in the molecular biology and biochemical steps involved in cloning and expression as well as in glycoprotein purification.

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The zona pellucida protein 3 (ZP3) is an essential component of the reproductive system as it functions as sperm receptor on the zona and as trigger of the acrosome reaction. To date, no recombinant human

5 ZP3 (rhZP3) with well-documented and characterized
biological activity is available. The aim of these
studies was to clone and express a biologically
active rhZP3 in human ovarian cells. A full-length
human ZP3 cDNA was generated by RT-PCR using mRNA
10 isolated from a human ovary. Sequencing of both
strands demonstrated identical composition to the
previously published cDNA sequence. An *in vitro*
transcription and translation system revealed a
protein core of 47 Kd for the product. To express
15 the human ZP3 *in vitro*, the ZP3 cDNA with a six-
histidine tail in its 3' end was inserted into a
pcDNA vector with a CMV promoter. The expression
construct was introduced into PA-1 cells by stable
transfection. The purification of rhZP3 was
20 performed using Wheat Germ Agglutinin, DEAE ion
exchange and Ni-NTA affinity chromatography.
Western blot analysis confirmed a molecular weight
of approximately 65 Kd for the secreted glycoprotein
which had a PI of 4.60 ± 0.05 . Glycosylation labeling
25 experiments demonstrated incorporation of ^3H -
galactose by the transfected cells. The rhZP3
demonstrated specific activities as ligand and
inducer of the acrosome reaction of live human
sperm.

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5 **SUMMARY OF THE INVENTION**

 The present invention provides a method to determine sperm activity comprising the steps of: (a) contacting an appropriate concentration of human
10 zona pellucida protein 3 with an appropriate amount of sperm under conditions permitting the formation of a complex between the human zona pellucida protein 3 and the sperm; and (b) determining the complex form.

15 The invention further provides A method to determine sperm activity comprising the steps of (a) contacting an appropriate concentration of human zona pellucida protein 3 with an appropriate amount
20 of sperm under conditions permitting an acrosome reaction to occur; and (b) determining the extent of the acrosome reaction.

 This invention also provides a diagnosis kit for
25 sperm activity comprising compartments with (a) an appropriate amount of human zona pellucida protein 3 and (b) the reagents used for establishing the conditions for allowing the binding of sperm.

30 Furthermore, this invention provides a diagnosis kit for sperm activity comprising compartments with (a) an appropriate amount of human zona pellucida protein 3 and (b) the reagents used for establishing the conditions for allowing an acrosome reaction.

35 Finally, this invention provides a diagnosis kit for sperm activity comprising three (3) compartments with (a) an appropriate amount of human zona pellucida protein 3; (b) the reagents used for
40 establishing the conditions for allowing the binding

5 DETAILED DESCRIPTION OF THE FIGURES

Fig. 1. Western Blot Analysis of protein sample from Caov-3, Caob-4, OVCAR-3, EB2, PA-1, SK-OV-3, and SW626 which were transfected with human ZP3
10 cDNA.

Fig. 2. Determination of expression of recombinant ZP3 in transfected PA-1 cells by RT-PCR. A) RT-PCR amplification of first strand of cDNA from the RNA
15 sample of PA-1 cells stable transfected with human ZP3 cDNA with primers A(CH1)/B(CH2) and A/C(B1). Location of PCR primers (Primer A 5' - TAGGATCCACCATGGACTGAGCTATAGG-3', Primer B 5' - TTATTCGGAAGCAGACACAGGGTGGGAGGCAGT-3', Primer C 5' -
20 TTCTCGAGTTAATGATGATGATGATGATGTTTCGGAAGCAGACACAGGGTGGG AGGCAGT-3').

Fig. 3. Protein Sample (rhZP3) from PA-1 cells stable transfected with human ZP3 cDNA and
25 solubilized zona (hZP3) was separated by SDS-PAGE. One of the gels was stained by Coomassie Blue. The other gel was analyzed by Western Blot. The human recombinant ZP3 has an identical molecular weight as the native ZP3 from the solubilized zona.

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Fig. 4. Study of the expression of recombinant human ZP3 using ELISA. Results are expressed as mean +/- SEM.

35 **Fig. 5.** Western blot analysis of recombinant human ZP3 isolated from WGA, DEAE and Ni-NTA columns. Western blot analysis of recombinant human ZP3 protein which was purified with WGA columns only(WGA), WGA and DEAE columns (DEAE) as well as

5 WGA, DEAE and Ni-NTA columns(Ni-NTA). The protein samples purified from non-transfected PA-1 cells (PA-1 without rhZP3) was used as a negative control.

Fig. 6. Determination of isoelectric point of rhZP3 using isoelectric focusing electrophoresis. A) Photo of rhZP3 on the isoelectric focusing gel. The protein sample isolated from the non-transfected PA-1 cells was used as a negative control. B) The computer graphics represent the photo of rhZP3 on the isoelectric focusing gel (A). C) Regression analysis of isoelectric point electrophoresis.

Fig. 7. ^3H -Metabolic Labeling Study of PA-1 cells with or without transfected human ZP3 cDNA. Results are expressed as mean \pm SEM.

Fig. 8. *In vitro* transcription and translation of human ZP3 cDNA. Human ZP3 cDNA was transcribed and translated *in vitro* by a reticulocyte lysate system from both 5' and 3' directions. A 47-kDa protein (indicated by arrow) was observed in the cDNAs only in 5' direction. A cDNA encoding luciferase was transcribed and translated as a positive control.

Fig 9. A glycoprotein sample (purified by sequential WGA-DEAE-Ni-NTA chromatography) from PA-1 cells transfected with human ZP3 (rhZP3) and human solubilized zona pellucida (hZP3) were separated by SDS-PAGE. Left gel: Coomassie staining of SDS denaturing gel. Right gel: Western blot analysis. The rhZP3 has an identical molecular weight as the native ZP3 from human solubilized zona pellucida.

5 **Figure 10.** Dose-dependent competitive inhibitory
effect of rhZP3 on sperm-zona binding in the HZA.
HZI: Hemizona index. Overall effect by ANOVA, $p < 0.0001$.

10 **Figure 11.** Dose-dependent induction of acrosomal
exocytosis of live sperm in suspension by rhZP3.
Overall effect by ANOVA, $p < 0.001$.

15 **Figure 12.** The acrosome reaction-inducing activity
of rhZP3 is mediated via pertussis toxin-sensitive G
proteins. Negative control 1: Ham's F-10 plus HSA;
negative control 2: culture medium from non-
transfected PA-1 cells; positive control: calcium
ionophore (5 μ M); rhZP3 tested at 500 ng/mL;
20 pertussis toxin (100 mg/mL) treated sperm then
tested with rhZP3 (500 ng/mL).

5 **DETAILED DESCRIPTION OF THE INVENTION**

As used herein, "human zona pellucida protein 3" is defined as a peptide or a polypeptide comprising the binding and/or the acrosome reaction inducement domain of the native human zona pellucida protein 3.

As appreciated by an ordinary skilled artisan, the amino acid sequences of the binding and/or the acrosome reaction inducement domain may be altered without affecting the binding and/or the acrosome reaction inducement activity. Accordingly, the term "human zona pellucida protein 3" covers any variation in the amino acid sequences of the binding and/or the acrosome reaction inducement domain without affecting the biological activities of the said domains.

The present invention provides a method to determine sperm activity comprising the steps of: (a) contacting an appropriate concentration of human zona pellucida protein 3 with an appropriate amount of sperm under conditions permitting the formation of a complex between the human zona pellucida protein 3 and the sperm; and (b) determining the complex form. In an embodiment, this invention provides the above method, wherein the concentration of the human zona pellucida protein 3 is 0.01 nanograms per ml to 10,000 nanograms per ml.

In a separate embodiment, the invention provides the above method, wherein the concentration is 0.01 nanograms per ml to 5,000 nanograms per ml. In another embodiment, the invention provides the above method, wherein the concentration is 0.01 nanograms per ml to 2,500 nanograms per ml. In yet another

5 embodiment, the invention provides the above method,
wherein the concentration is 0.01 nanograms per ml
to 1,000 nanograms per ml. In another embodiment,
this invention provides the above method, wherein
the concentration is 0.01 nanograms per ml to 500
10 nanograms per ml.

In a separate embodiment, this invention provides
the above method, wherein the concentration is 0.01
nanograms per ml to 100 nanograms per ml. In still
15 another embodiment, this invention provides the
above method, wherein the concentration is 0.01
nanograms per ml to 30 nanograms per ml. In a
separate embodiment, the invention provides the
above method, wherein the human zona pellucida
20 protein 3, or the sperm, is fixed on a matrix.

The invention further provides a method to determine
sperm activity comprising the steps of (a)
contacting an appropriate concentration of human
25 zona pellucida protein 3 with an appropriate amount
of sperm under conditions permitting an acrosome
reaction to occur; and (b) determining the extent of
the acrosome reaction.

30 In an embodiment, this invention provides the above
method, wherein the concentration of the human zona
pellucida protein 3 is 0.01 nanograms per ml to
10,000 nanograms per ml. In a separate embodiment,
the invention provides the above method, wherein the
35 concentration is 0.01 nanograms per ml to 5,000
nanograms per ml. In another embodiment, the
invention provides the above method, wherein the
concentration is 0.01 nanograms per ml to 2,500
nanograms per ml.

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In yet another embodiment, the invention provides the above method, wherein the concentration is 0.01 nanograms per ml to 1,000 nanograms per ml. In another embodiment, this invention provides the above method, wherein the concentration is 0.01 nanograms per ml to 500 nanograms per ml. In a separate embodiment, this invention provides the above method, wherein the concentration is 0.01 nanograms per ml to 100 nanograms per ml.

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In still another embodiment, this invention provides the above method, wherein the concentration is 0.01 nanograms per ml to 30 nanograms per ml. In a separate embodiment, the invention provides the above method, wherein the human zona pellucida protein 3, or the sperm, is fixed on a matrix.

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This invention also provides a diagnosis kit for sperm activity comprising compartments with (a) an appropriate amount of human zona pellucida protein 3 and (b) the reagents used for establishing the conditions for allowing the binding of sperm.

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Furthermore, this invention provides a diagnosis kit for sperm activity comprising compartments with (a) an appropriate amount of human zona pellucida protein 3 and (b) the reagents used for establishing the conditions for allowing an acrosome reaction.

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Finally, this invention provides a diagnosis kit for sperm activity comprising three (3) compartments with (a) an appropriate amount of human zona pellucida protein 3; (b) the reagents used for establishing the conditions for allowing the binding

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5 of sperm; and (c) the reagents used for establishing the conditions for allowing an acrosome reaction.

First Series Of Experiments

10 **Materials and methods**

Isolation of human ovarian mRNA and construction of cDNA for hZP3

Total RNA was isolated from the human ovary (the utilization of human tissue was approved by the Institutional Review Board of Eastern Virginia Medical School) by using the guanidium thiocyanate method (Chirgwin, et al, 1979). A pair of primers was designed based on the published sequence of hZP3 cDNA with additional restriction enzyme sites and histidine tail (Chamberlin and Dean, 1990). The sense primer was located between base 1 to 22 with Bam HI site in the 5' end (5'-TAGGATCCACCATGGAGTGAGCTATAGG-3'). The antisense primer was located between base 1256 and 1262 (5'-TTCTCGAGTTAATGATGATGATGATGAGATGTTTCGGAAGCAGACACAGGGTGGAGGCAGT-3'). A sequence of Xho I restriction site and a sequence coding for six histidine residues were introduced into 5' end of this primer for the purpose of the purifying the recombinant protein as well as for subcloning. RT-PCR of the mRNA samples from human ovaries revealed a single band of approximately 1,300 bases. This PCR product was purified and inserted into a mammalian cell expression vector, pcDNA 3.1 (Invitrogen, Carlsbad, CA). The positive clone was sequenced and found to be identical to those of the published hZP3 (Chamberlin and Dean, 1990).

5 *Stable-transfection of PA-1 cells with hZP3 cDNA*

PA-1 cells (American type Culture Collection, ATCC, Rockville, MA) were selected for stable-transfection of hZP3 cDNA. The cells were grown in MEM medium (Sigma, St. Louis, MO) supplemented with 5% Fetal Bovine Serum (FBS). When the cells reached 70% confluence, the medium was changed and was transfected with 5 µg of purified hZP3 cDNA by calcium precipitation method (Sambrook et al, 1989). After 24 hours, the cells were washed three times to remove the calcium and continued to culture in the MEM medium for an additional 24 hours. Two milligrams per milliliter of neomycin were used to select the cells stable-transfected with hZP3. Approximately 10 single colonies of stable-transfected PA-1 cells were obtained. To examine whether hZP3 was expressed in these cells, RT-PCR was used with 2 primers specific to hZP3 as described above.

25 *Cell culture and medium collection*

PA-1 cells were routinely cultured in MEM medium supplemented with 5% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every two days. After two weeks, cell number was amplified and cells were transferred from 100mm culture dishes to 150mm cell culture dishes. MEM containing 50% FBS and 5% DMSO was used for freezing the transfected PA-1 cells (at -196°C in liquid nitrogen). Since human ZP3 is a secretion protein, the culture medium from the stable transfected PA-1 cells was collected for further purification. Twenty-five dishes (150 mm) were cultured and 20 ml of medium were collected from each plate every 48

5 hr. The collected medium was then centrifuged at
1000 g for 10 minutes to remove cellular debris and
stored at 4°C with the addition of protease
inhibitors (100µg/ml PMSF, 2µg/ml Leupeptin, 1µg/ml
Pepstatin and 2mM EDTA). Glycoprotein purification
10 was performed within a one-week period.

Sequential affinity chromatography

A sequential procedure combining WGA (*Wheat Germ
Agglutinin*) (Vector laboratories, Burlingame, CA),
15 DEAE ion exchange and Ni-NTA (nitrilo-tri-acetic
acid, Qiagen) affinity chromatography was developed
to successfully purify bioactive rhZP3. The
collected medium was first passed through a 10 ml
agarose-bond WGA (Vector Laboratories, Burlingame,
20 CA) column at the rate of three resin volumes per
hour at 4°C, to initially achieve glycoprotein
separation. The glycoproteins bound to the WGA
resin were eluted with WGA elution buffer (10mM PBS,
0.15M NaCl, 0.5M N-acetyl-D-glucosamine, pH 7.4).

25 The eluted glycoproteins were dialyzed against DEAE
cellulose binding buffer (5 mM PBS, pH 8.0)
overnight at 4°C. The glycoprotein fraction isolated
from the previous affinity chromatography was
30 applied to the DEAE-Cellulose column (1x5 cm) at the
flow rate of three to four resin volumes per hour.
The bound protein was eluted with the same binding
buffer with pH gradient from pH 8.0 to pH 3.5.
Human recombinant ZP3 was eluted out between pH 6 to
35 pH 4. The fractions containing recombinant hZP3
were dialyzed against Ni-NTA binding buffer (50mM
PBS, 300mM NaCl, pH 8.0) overnight at 4°C. The
dialyzed glycoproteins were then applied to the Ni-

5 NTA column at a flow rate of three to four resin
volumes per hour. The specific bond glycoproteins
were eluted with binding buffer containing different
concentrations of imidazole. The purified
glycoprotein was either used immediately upon
10 collection for testing of biological activity or
stored at -20 °C.

Western blotting

The protein samples, purified by sequential affinity
15 chromatography, were separated with 8% SDS-PAGE gels
using Hoefer SE 220 minigel electrophoresis
apparatus (Hoefer). An anti-ZP3 antiserum was
purchased from Cocalico Biologicals, Inc.
(Reamstown, PA). This decapeptide is a conserved
20 epitope among different mammalian ZP3 including the
human. The antiserum was produced by immunizing a
rabbit with the synthetic decapeptide D-V-T-V-G-P-L-
I-F-L (Hinsch et al, 1994) which was linked to
keyhole limpet hemacyanin (KLH). Wet transfer of
25 proteins from gel to hybond ECL nitrocellulose
membrane (Amersham) was performed at 100 Volts for
2.5 hours at 4°C with the transfer buffer (25 mM
Tris-HCl; 192 mM glycine (Fisher); 20% methanol, pH
8.3). After transferring, the nitrocellulose
30 membrane was blocked with blocking buffer (80% Tris-
HCl buffer, pH 7.5; 15% H₂O; 5% BSA) at room
temperature for 3 hours by gently shaking. The
rabbit anti-ZP3 antiserum was used as primary
antibody which was diluted at 1: 1000 in solution A
35 (80% Tris-HCl, pH 7.5; 20% BSA). Blocked
nitrocellulose membrane was incubated in the primary
antibody solution at room temperature for one hour
with gentle shaking. The nitrocellulose membrane

5 was washed with washing buffer A (PBS containing
0.4% Tween 20). The secondary antibody [goat anti-
rabbit IgG-HRP antibody conjugate (Amersham)] was
diluted by the washing buffer A at 1:2000 dilution.
After washing with washing buffer, the
10 nitrocellulose membrane was incubated in secondary
antibody solution at room temperature for one hour.
The membrane was washed with washing buffer B (PBS
containing 0.3% Tween 20) for five minutes three
times with fresh changes which was followed by
15 washing buffer C (PBS containing 0.1% Tween 20) for
five minutes three times with fresh changes of the
washing buffer. The nitrocellulose membrane was
exposed to the detection solution [detection reagent
1 and detection reagent 2 (1:1, v/v, Amersham)].
20 The membrane was placed with protein side face to
film and exposed in the film cassette for 30 to 60
seconds. The film was developed with a Konica
developing machine.

25 *Metabolic labeling experiment*

In order to label the new synthesis of glycoproteins
by the PA-1 cells, a ^3H -galactose metabolic labeling
experiment was performed (Lennarz and Hart, 1994).
PA-1 cells stable-transfected with or without hZP3
30 cDNA were cultured for 4 hours until attached to the
plate surface, and then washed by 1xPBS to remove
the cell-debris. A "radioactive" medium containing
250 μCi of ^3H -galactose was added and cultured for
additional 16 hours. The medium was collected and
35 the unincorporated ^3H -galactose was removed from the
collected medium with Centriprep with a 30 kDa cut-
off range (1,500 \times g for 30 min.). The remnant was
washed three times with PBS buffer. Both the

5 remnant and the washing solutions were collected for
further analysis. The remnant was concentrated
using Centricon until the final volume was 2.0 ml
and passed through a WGA lectin column prepared as
described previously. The WGA column was washed
10 with ten bed volumes of WGA washing buffer, and
eluted by elution buffer. Both WGA bound and non-
bound fractions were collected respectively and
loaded in SDS-PAGE gel with 4% stacking gel and 8%
separation gel. The gel was then dried and exposed
15 to an X-ray film.

Hemizona assay

Hemizona assay was conducted to demonstrate the
binding activity of rhZP3 to human sperm (Burkman et
20 al, 1988; Oehninger et al, 1990, 1995). An inverted,
phase-contrast microscope equipped with a
micromanipulation system was routinely employed to
cut the oocyte into halves to obtain matching
hemizonae. Oocytes used in the experiments were
25 obtained from surgically-removed ovarian tissue or
discarded from IVF therapy under approval of the
Institutional Review Board at Eastern Virginia
Medical School. The hemizonae were washed in PBS to
completely deplete the cytoplasm. Sperm samples were
30 from healthy fertile donors. A swim-up procedure
was applied to obtain motile sperm, which were then
adjusted to 0.5 million/ml in Human Tubal Fluid
(HTF) supplemented with 0.3% Human Serum Albumin
(HSA) for hemizona assay. In one Petri dish, one
35 droplet of 100 ul of sperm suspension was placed as
a control. Another droplet of sperm was pre-treated
with rhZP3. One hemizona was placed into the
control droplet and the matching hemizona was placed
in the sperm droplet treated with rhZP3. Five pairs

5 of hemizona were used for each experiment. All dishes were incubated at 37°C, 5% CO₂ for 4 hours. Each hemizona was removed and rinsed 15 times in PBS, and transferred to the counting dish. The number of sperm bound to the surface of each
 10 hemizona was counted under phase microscopy. The HZI was calculated to evaluate rhZP3-binding activity (competitive inhibition) as follows: number of sperm bound for treatment/number of sperm bound for control x 100.

15

Assessment of acrosome reaction

The purified rhZP3 was also applied to test its activity to induce human sperm acrosome reaction. The motile sperm obtained by swim-up procedure were
 20 allowed to capacitate in HTF/ 0.5% HSA for four hours at 37°C in 5% CO₂ in humidified air. The motile sperm concentration used to detect acrosome reaction was set at 2 million /ml. A series of 100 ul of capacitated sperm aliquots with different inducers
 25 were prepared in Eppendorf vials and cultured in the incubator in 95% air, 5% CO₂, at 37°C for 30 minutes. The test was conducted as follows: positive control: calcium ionophore A23187 (Sigma) at 5 µM; negative controls: 1) sperm culture medium alone, and 2)
 30 protein isolated from the culture media of non-transfected PA-1 cells (NT); test: rhZP3. Triple slides were made for each assay. Hoechst 33258 stain was used for determination of sperm viability. Fluorescein isothiocyanate conjugated Pisum sativum
 35 agglutinin (FITC/PSA) (Sigma) staining technique was employed to evaluate the acrosome reaction (Cross et al, 1986). Blind reading was required for evaluation and at least 100~200 cells from 5 random

5 fields were evaluated per spot on the slide. Spermatozoa demonstrating no fluorescence over sperm head or only fluorescence at the equatorial region were considered to be acrosome-reacted. The results were expressed as percentage of acrosome-reacted
 10 spermatozoa in the total population counted (Cross, 1986).

In some experiments, Solubilized zona pellucida were used as control to test its function to induce sperm
 15 acrosome reaction. The solubilized zona pellucida were prepared according to Franken et al (1996). Ooplasm was removed using a small glass micropipette. A vigorous pipetting action would break their zona causing ooplasm to be spilled into
 20 surrounding medium. The chosen amount of zonae was transferred into an eppendorf tube and was centrifuged for 15 minutes at 300xg. Using a stereomicroscope, the media were removed with pipette, making sure not to disturb zona. The final
 25 result was to remove maximum medium. A chosen volume (depending on the zona concentration needed) of 10 mM HCl was added. Under the microscopic vision, zona /HCl was pipetted up and down until all zona were dissolved. Then 10 mM NaOH of equal
 30 volume as the HCl was added and mixed well to obtain the stock zona solution.

Results

In vitro expression of recombinant human ZP3 in PA-1
 35 cells

A full-length human ZP3 cDNA was generated by RT-PCR using mRNA isolated from human ovarian cells. A 1,278 bp DNA fragment (full length of human ZP3

5 cDNA) was obtained after PCR amplification and
further characterized by restriction mapping,
Southern blot analysis and sequencing of both
strands demonstrated identical composition to the
published sequence (Chamberlin and Dean, 1990). In
10 addition, the use of an in vitro transcription and
translation system (reticulocyte lysate)
demonstrated the expression of a 47 KD protein, the
exact molecular weight as predicted from the DNA
sequence (Dong et al, 2000).

15 In order to obtain high levels of expression of ZP3
in mammalian cells, the ZP3 cDNA was inserted into a
pcDNA3.1 vector (Invitrogen, Carlsbad CA) with a CMV
promoter. To insure biological activity of ZP3, the
20 human ovarian cell was used to express the
recombinant ZP3. Seven human ovarian cell lines
(EB2, Caov-3, Pa-1, Caob-4, OVCAR-3, SK-OV-3, and
SW626) were purchased from ATCC (Rockville, MD) and
transiently transfected with pcDNA/ZP3 expression
25 construct. After several trials only PA-1 cells
were found to exhibit high levels of expression of
ZP3 with biological activity (Fig 1). The pcDNA/ZP3
expression construct was transferred into PA-1 cells
and treated with neomycin for selection of stable
30 transfection. After three months of treatment, ten
positive clones were selected. RT-PCR of the mRNA
isolated from these clones, with human ZP3's
specific primers, displayed high expression levels
of human ZP3 (Fig. 2). ELISA analysis using the
35 polyclonal anti-human ZP3 (anti-decapeptide
antiserum) demonstrated expression of rhZP3 by the
cells. One of the ten positive clones was chosen for
subsequent study, as it expressed the highest levels
of ZP3 with biological activity. Western Blot

5 analysis of this protein reveals that it has an identical molecular weight, approximately 65KD, as native human ZP3 from the solubilized zona (Fig 3).

10 In order to study the expression level of hZP3 in the transfected PA-1 cell, ELISA assay was carried out. Fig. 4 shows that recombinant human ZP3 was detectable in three hours (data not shown), reaching to its peak in twenty-four hours. The recombinant human ZP3 production gradually decreased after
15 forty-eight hours.

Isolation and purification of recombinant human ZP3

Since human ZP3 is a glycoprotein, a wheat germ agglutinin (WGA) column was used to carry out the
20 first isolation. These isolated glycoproteins were further purified using DEAE-ion exchange and Ni-NTA affinity chromatography. Approximately 3 to 5 mg of recombinant ZP3 containing glycoprotein was isolated from one liter of media (Fig 5). Since six
25 histidines have been added to the C-terminal of rhZP3, a Ni-NTA column was used to further purify the recombinant ZP3. Western blot analysis of this Ni-NTA mediated purification displayed a high purity of human ZP3. In parallel experiments, the protein
30 samples were also analyzed with SDS-PAGE electrophoresis, and stained with Coomassie Blue. According to densitometer scanning analysis, rhZP3 accounted for 80% to 90% of the total purified proteins. Thus, 1 milligrams to 1.5 milligrams of
35 rhZP3 was finally purified from one liter of culture medium (Fig 6).

5 *Measuring the isoelectric point of recombinant human ZP3*

For further biochemical analysis, isoelectric focusing electrophoresis was performed. Approximately five microgram of recombinant ZP3 was
 10 loaded into an isoelectrophoresis tube with ampholytes (pH 3-10) in a wide range, and ampholytes (pH 4-8) in a narrow range. The same amount protein sample isolated from the PA-1 cells without transfected with hZP3 cDNA was used as a control. As
 15 shown in Fig. 6, recombinant human ZP3 had an isoelectric point of 4.60 ± 0.05 .

Determination of glycosylation by ^3H -galactose metabolic labeling experiment

20 In order to study if the new synthesis of recombinant ZP3 by the PA-1 cells is glycosylated, a ^3H -galactose metabolic labeling experiment was carried out. Fig 7 reveals that the PA-1 cell without stable-transfected with hZP3 has relatively
 25 low incorporation ^3H . However, after stable-transfection with hZP3 cDNA, the relative incorporated radioactivity dramatically increased, thus indicating that a large amount of new synthesis protein was glycosylated. Electrophoresis of the
 30 product of this ^3H -galactose metabolic labeling product has demonstrated that a great amount of ^3H labeled protein was crowded at the regions near 65 KD (data not shown)

35 *Testing the sperm binding activity of recombinant human ZP3 by the hemizona assay*

Sperm culture medium (HTF/0.5%HSA) or protein isolated from medium collected from PA-1 cells which

5 were not transfected with ZP3 cDNA (NT) was used as
the internal control in each experiment. Hemizona
assay results demonstrated a dramatic decrease of
sperm-ZP binding when sperm were pre-incubated for
30 min with rhZP3 (approximately 60% inhibition at
10 30 ng/ml)). These data demonstrated that rhZP3
effects a specific and potent competitive inhibition
of sperm binding to the homologous zona pellucida
(Table 1).

15 **Table 1. Hemizona index for testing of recombinant
human ZP3 (rhZP3)**

N=5 semen donors × 5 pairs of matching hemizonae per sample

Sperm exposed to test reagent	Sperm exposed to control conditions	HZI (Mean ±SEM)	p-value (paired t-test)
30 ng/ml rhZP3	HTF/0.5% HSA	43.6 (3.3)	<0.01
30 ng/ml rhZP3	30 ng/ml NT	44.5 (3.6)	<0.01
30 ng/ml NT	HTF/0.5% HSA	94.2 (0.3)	>0.5

20 *Analysis of the ability of recombinant human ZP3 to
induce sperm acrosome reaction*

The analysis of the acrosome reaction observed in
response to purified rhZP3, natural solubilized
human ZP and the calcium ionophore revealed that all
agonists enhanced the percentage of acrosome reacted
25 sperm when compared to control conditions (i.e.,
culture medium alone or protein purified from non-
transfected PA-1 cells) (Table 2). First the time
course study of sperm capacitation was conducted.
After different period of capacitation time, sperm
30 were treated with rhZP3 (30ng/ml) for thirty minutes
to trigger acrosome reaction. The results indicated
that there was a trend of increase in the percentage

5 of acrosome-reacted sperm as the capacitation time
 prolonged (data not shown) up to 8~10 hours. In our
 ongoing series of experiments, 4 hour capacitation
 was used because at this time frame the sperm are
 99% alive as compared to 86%, 82% and 66% of live
 10 sperm for 8, 12 and 24 hours of capacitation time
 respectively. So all experiments were performed
 after 4 hours of capacitation. The rhZP3 increased
 the percentage of acrosome-reacted by 150% from
 control conditions after 30 minutes of pre-
 15 incubation with sperm at 30 ng/ml. [This activity is
 much stronger than that reported for the CHO cell
 product which induced a similar percentage of
 acrosome reaction in human sperm following 24 hour
 sperm capacitation at a dose of 15-20 ng/ μ l (van
 20 Duin et al, 1994).]

Table 2. Analysis of the percentage of acrosome-reacted sperm by FITC-PSA.

	Negative control	Non-transfected (NT, 30 ng/ml)	Calcium ionophore (5 μ M)	rhZP3 (30ng/ml)	Solubilized zona (0.5ZP/ μ l)
% acrosome reacted-sperm	7.7 (3.2)	9.5 (2.4)	22.5 (4.1)*	18.3 (1.4)*	14.8 (6.2)*
% sperm viability	95.1 (2.6)	98.0 (1.0)	96.5 (2.0)	96.7 (3.2)	97.2 (2.5)

25 *P < 0.05 compared to control conditions

Mean (\pm SEM), n= 29 ejaculates from 11 different donors, 3 different purification
 lots of rhZP3.

5 **Discussion**

ZP3 is an essential protein in the reproductive system. Because of the difficulty in obtaining human ZP3 from native sources, the mechanism(s) throughout which ZP3 is involved in human fertilization as well as the molecular structure of human ZP3 are poorly understood. Using molecular and cell biology technologies, several groups have attempted to produce biologically active recombinant human ZP3. Analysis of the current knowledge indicates that no rhZP3 with well-documented and characterized biological activities is available. Because human ZP3 has a strong hydrophobic protein backbone (Chamberlin and Dean, 1990), as well as probably large carbohydrate side chains, the glycoprotein is extremely difficult to be produced by recombinant DNA technology. Some groups have expressed ZP3 in E.Coli; this results in a low-soluble and non-glycosylated ZP3 protein (Champan and Barratt, 1996). Other groups have also attempted a cell-free translation of ZP3, also resulting in incomplete biological activity human ZP3 (Whitmarsh, et al, 1996). Other groups have used CHO cells to express ZP3 (van Duin et al, 1994). This recombinant hZP3 displayed an acrosome reaction-inducing activity only at very high levels of recombinant protein (15 to 20 µg/ml) after a long preincubation time (maximal effects observed after 18 hours). Furthermore, this recombinant protein did not demonstrate any binding activity to human sperm; therefore, it is considered that this protein only has partial biological activity.

5 Using RT-PCR we have generated a full length human
ZP3 from the mRNA isolated from human ovary. DNA
sequencing analysis of the cDNA revealed that it is
identical to the published sequence (Chamberlin and
Dean, 1990). In addition, the use of an *in vitro*
10 transcription and translation system (reticulocyte
lysate) demonstrated the expression of a 47 KD
protein, the exact molecular weight as predicted
from the DNA sequence (Dong et al, 2000).

15 Optimal glycosylation is a crucial step to produce a
biologically active rhZP3. The carbohydrate side
chains are important to provide solubility of the
protein, and also appear to be essential for the
binding activity for rhZP3. O-linked has been
20 demonstrated to be required for the binding of mouse
sperm to zona pellucida (Florman et al, 1985). Up
to now there is no efficient way to modify the
glycosylation of ZP3 under *in vitro* conditions.
Therefore, selecting an expression system for the
25 production of recombinant human ZP3 with correct
glycosylation is extremely important. We believe
that since glycosylation is tissue- and species-
specific, expression of ZP3 cDNA in a human ovarian
cell line could produce recombinant human ZP3 with
30 full biological activity. As described in the
result section, we initially tried several available
human ovarian cell lines. After a long period of
study, we discovered that only PA-1 cells could
express the biologically active recombinant ZP3.
35 Never before has a recombinant ZP3 protein have been
shown to possess both the ability to bind to human
sperm, as well as the ability to induce the acrosome
reaction; yet through our studies, we have been able
to generate a recombinant protein with both those

5 abilities. Interestingly, Whitmarsh et al (1996)
 showed that with their in vitro transcription-
 translation system, their recombinant ZP3 (rZP3)
 supposed to be without glycosylation could have
 binding activity to sperm using bead coated with
 10 rZP3; they also reported that rZP3 from CHO cells
 could induce sperm acrosome reaction.

The biochemical studies using a cell-free
 translation system have demonstrated that this
 15 protein has a collect size of protein backbone (47
 KD). Western blot analysis has demonstrated that
 this recombinant protein has approximately 65 KD,
 thus agreeing with the native human ZP3 (Shabanowitz
 et al, 1988). The result from these studies reveals
 20 that an approximately 18 KD difference between
 glycosylated recombinant protein and the ZP3 protein
 backbone may result from the glycan side chains.
 Furthermore, the metabolic labeling study has
 demonstrated that the PA-1 cells transfected with
 25 ZP3 expression vector produces a great amount of
 newly synthesized glycoprotein. All of these data
 strongly suggest that PA-1 cells can glycosylate the
 ZP3 protein backbone. More importantly, our rhZP3
 not only can induce the acrosome reaction in
 30 capacitated human sperm, but can also function as a
 ligand to human sperm. Furthermore, our initial
 studies demonstrated that these biological
 activities display a dose-responsive pattern. (Dong
 et al, 2000)

35 Isoelectrical point studies have shown that the
 rhZP3 has PI values approximately near pH 5.60,
 suggesting that our recombinant ZP3 produced by the
 PA-1 cells may have different degrees of

glycosylation. As control, we have used a protein sample that was collected from PA-1 cells not transfected with the hZP3 cDNA; these samples were purified by the sequential affinity chromatographic procedures used to purify rhZP3. This control sample displayed a different pattern (with PI values approximately near pH 5.4) from that of human recombinant ZP3; thus rejecting possibilities of contamination with other secretion proteins from PA-1 cells. Since glycosylation is a major contribution to the PI value of glycoprotein, our ZP3 shows similarities with the native ZP3 (PI value) suggesting that rhZP3 may have a similar glycoside chain pattern as native human ZP3.

Our rhZP3 demonstrated ligand activity by competitively inhibiting sperm-zona pellucida binding in the HZA. The HZA is a useful tool to examine the mechanisms of initial sperm-oocyte interaction by providing a homologous, internally controlled test that assesses the specific, irreversible and tight binding of sperm to the zona pellucida as well as the zona-induced acrosome reaction (Oehninger, 1990). To the best of our knowledge, this is the first time that a rhZP3 has been proven to competitively inhibit sperm-zona pellucida binding in a controlled fashion. Our rhZP3 also demonstrated a potent ability to induce the acrosome reaction in live spermatozoa. Here, we have proven that this effect is capacitation-dependent. Previously, we demonstrated that both ligand and acrosome reaction-inducing activities are dose-dependent, with maximal effects in the range of 30-1,000 ng/ml (Dong et al; 2000). This activity is much stronger than that reported for the CHO cell

5 product which induced a similar percentage of
acrosome reaction in human sperm following 24 hour
sperm capacitation at a dose of 15-20 ng/ μ l (van
Duin et al, 1994). The level of induction of
10 acrosomal exocytosis was similar to the one observed
for two well-known agonists used as positive
controls; i.e., a calcium ionophore and solubilized
human zona pellucida. The structural features of
acrosome-reacted spermatozoa (assessed by
transmission electronmicroscopy) also showed
15 similarity to the acrosomal exocytotic changes
observed with the control agonists (Dong et al,
2000).

In the zona pellucida, ZP3 associates with ZP2 and
20 ZP1 to form a network structure (Wassarman, 1988).
This network structure prevents aggregation of these
glycoproteins. However, in solution these
glycoproteins tend to aggregate together. This
phenomenon has been observed in several recombinant
25 glycoproteins, including ZP3 (Champan and Barratt,
1997). In purifying rhZP3, we have avoided this
problem by obtaining a highly purified product, and
testing its biological activities within a week-
period while maintaining the protein at 4°C. However,
30 we have found similar problems of aggregation of the
glycoprotein as we attempted freezing the product.
This problem becomes worse as larger amounts of the
glycoprotein are trying to be produced. Different
strategies are being looked into in order to produce
35 and purify large amounts of biologically active
rhZP3 in our laboratory.

5 In summary, using a human ovarian cell line, we have
successfully cloned and expressed, and purified a
biologically active recombinant human ZP3. This
protein has a molecular weight of 65KD, with an PI
in the range of 4.6 ± 0.05 . In vitro translation by a
10 cell-free system and ^3H metabolic labeling
experiments revealed that our recombinant ZP3 has a
large glycan side chain (approximately 18KD).
Importantly, the present data and the results of our
previous studies (Dong et al, 2000) unequivocally
15 present evidence showing that the glycoprotein has
biological activity as it acts as ligand to human
sperm and induces the acrosome reaction. The
complete biochemical and functional characterization
of this recombinant human ZP3 (rhZP3) may allow us
20 in the future to (1) further examine the physiology
of human gamete interaction including the
identification of the putative receptor(s) on the
surface of human sperm; (2) develop new assays to
test for male infertility; and (3) investigate new
25 contraceptive strategies.

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5 Second Series Of Experiments

Objectives: To clone and express a recombinant human ZP3 (rhZP3) and to characterize its biological activities as sperm ligand and inducer of the acrosome reaction.

Study design: Human ovarian teratocarcinoma (PA-1) cells were transfected with an expression vector containing human ZP3 cDNA with a sequence coding for a six histidine tail introduced into its 3' end. Purification of the secreted glycoprotein was performed by sequential affinity (lectin and nickel-nitrilotriacetic) and ion exchange chromatography.

Results: Western blot analysis confirmed a molecular weight of approximately 65 kDa of the purified product. A cell-free translation system revealed a correct size protein backbone of 47 kDa. The rhZP3 demonstrated specific, potent and dose-dependent competitive inhibition of sperm-zona pellucida binding *in vitro* under hemizona assay conditions. Recombinant hZP3 also stimulated the acrosome reaction of live sperm. This effect was fast, dose- and capacitation time-dependent. Furthermore, preincubation with pertussis toxin, an inactivator of heterotrimeric G proteins blocked rhZP3-induced acrosomal exocytosis.

Conclusion: The rhZP3 expressed in PA-1 cells manifests the full spectrum of expected biological activities and, therefore, represents a valuable tool for examination of human fertilization and the

5 design of new strategies in diagnostic male infertility and contraception.

Extensive work in the murine model has resulted in the identification and isolation of the primary
10 receptor for sperm located at the level of the zona pellucida, a glycoprotein called zona pellucida protein 3 (ZP3) (1,2). Sperm binding to the zona pellucida is supported by the interaction of ZP3 and putative complementary binding protein(s) present in
15 the sperm plasma membrane. In the mouse, bound sperm undergo the acrosome reaction (triggered by ZP3) and start penetration of the zona matrix (1,2). It has been proposed that GTP-binding proteins (G_i class) function as a signal transducer element distal to
20 ZP3-mediated interactions (3). ZP3-activated, heterotrimeric G proteins (pertussis toxin-sensitive) may stimulate changes in ionic conductance and/or a variety of intracellular second messenger systems resulting in acrosomal exocytosis
25 (3).

Recently, full-length cDNA clones of ZP3 for different mammalian species have been isolated (reviewed in 4). Cloning cDNAs encoding ZP3 has made
30 the expression of recombinant ZP3 in tissue culture cell lines possible and represents a potential option to obtain large amounts of ZP3. The expression of recombinant ZP3s has been reported, at least in the mouse and human (2,5-8). In the human,
35 recombinant ZP3 has been expressed using several approaches; i.e., *Escherichia coli*, in vitro transcription and translation systems, Chinese hamster ovary (CHO) cells and in African green monkey kidney (COS) cells (reviewed in 6-8).

5 However, full biological activity of a human product has not been demonstrated, which is possibly due to inadequate or incomplete glycosylation of the recombinant protein (6,7).

10 The production of a purified, glycosylated recombinant human ZP3 (rhZP3) in a biologically active form is fraught with technical difficulties. In vitro transcription and translation systems and expression in *Escherichia coli* have lead to the
15 production of recombinant products showing variable acrosome reaction-inducing activity (6,7). However, no direct or specific sperm-ligand capacity using homologous sperm-zona pellucida binding bioassays has been reported for such non-glycosylated
20 products. In addition, protein solubility has been a major difficulty encountered. The CHO cell product has been shown to possess acrosome reaction-inducing activity. However, no data are available related to sperm binding in validated assays (6-8).

25 Male infertility is present in 30% to 50% of childless couples and may represent the commonest single defined cause of infertility (9). Defects of sperm-zona pellucida interaction can be diagnosed in
30 a high proportion of infertile men in the presence or absence of abnormalities of the basic sperm parameters and are associated with fertilization failure in assisted reproduction (10,11). Although sperm-zona pellucida binding and acrosome reaction
35 bioassays have been validated in the clinical arena (10-14) the development of simpler, more standardized and universally applicable diagnostic methods are warranted. A biologically active, rhZP3 might prove to be instrumental in such an endeavor.

5 The objectives of these studies were: (1) to clone
and express human ZP3; and (2) to characterize the
biological properties of the recombinant product as
sperm ligand and inducer of acrosomal exocytosis.
For these purposes, we cloned and expressed the cDNA
10 of human ZP3 by stable transfection in a human
ovarian cell line (PA-1 cells). We selected this
cell line since glycosylation is tissue- and
species- specific and because the PA-1 cells have
been successfully used as an expression host to
15 express other glycosylated native proteins, such as
lactosaminoglycan-carrier glycoprotein, heparin-
binding protein and recombinant fibronectin (15). We
purified the recombinant glycoprotein product and
characterized its biological activities using
20 validated bioassays. We further investigated whether
rhZP3 induction of acrosomal exocytosis is mediated
via signaling cascades involving activation of
heterotrimeric G proteins.

25 MATERIALS AND METHODS

These studies were approved by the Bio-safety
Committee and by the Institutional Review Board at
Eastern Virginia Medical School.

30 *Isolation of human ovarian mRNA and construction of
cDNA for human ZP3*—Total RNA was isolated from the
human ovary by using the guanidinium thiocyanate
method. A pair of primers was designed based on the
35 published sequence of hZP3 cDNA with additional
restriction enzyme sites and a histidine tail (12).
The sense primer is located between bases 1 to 22
with Bam HI site in the 5' end (5'-
TAGGATCCATGGAGCTGAGCTATAGGC-3'). The antisense

5 primer is located between base 1256 and 1262 (5'-
 TTCTCGAGTTAATGATGATGATGATGATGTTTCGGAAGCAGACACAGGGTGGG
 AGGCAGT -3'). A sequence of Xho I restriction site
 and a sequence coding for six histidine residues
 10 were introduced into 5' end of this primer for the
 purpose of the purifying the recombinant protein as
 well as for subcloning. Reverse transcription-
 polymerase chain reaction (RT-PCR) of the mRNA
 samples from the human ovary revealed a single band
 of approximately 1,278 bp. This PCR product was
 15 further characterized by restriction mapping,
 Southern blotting and sequencing analysis
 demonstrating identical composition to the published
 human ZP3 (16). The PCR product was inserted into a
 mammalian cell expression vector, pcDNA 3.1
 20 (Invitrogen, Carlsbad, CA). An in vitro
 transcription and translation system (Reticulocyte
 Lysate System; Promega, Madison, WI) was used to
 determine the molecular weight of the (non-
 glycosylated) protein core of the recombinant ZP3.

25 *Stable-transfection of PA-1 cells with human ZP3*
cdNA-PA-1 cells (human ovarian teratocarcinoma
 cells, American Type Culture Collection; Rockville,
 MA) were grown in MEM (Minimal Essential Medium;
 30 Sigma Chemical Co., St. Louis, MO) supplemented with
 5% Fetal Bovine Serum (FBS; Sigma). The cells were
 transfected with purified hZP3 cDNA using the
 calcium phosphate precipitation method. Neomycin
 was used to select the cells stable-transfected with
 35 human ZP3. After collection, the cell culture
 medium was centrifuged at 1000 g for 10 minutes to
 remove cellular debris and stored at 4°C with the
 addition of protease inhibitors (100µg/ml
 phenylmethylsulfonyl, 2µg/ml leupeptin, 1µg/ml

5 pepstatin and 2mM ethylenediaminetetraacetic acid; Sigma). The medium was maintained for no more than 5 days before glycoprotein purification and testing of biological activity.

10 Sequential chromatography—The collected medium was first passed through an agarose-based WGA column (Wheat Germ Agglutinin; Vector, Burlingame, CA) at the flow rate of three resin volumes per hour at 4°C, to initially achieve glycoprotein separation. The
 15 resin was washed with a buffer (10mM PBS, 0.15M NaCl, pH 7.4) until the flow-through A_{280} was less than 0.01. The glycoproteins bound to the WGA resin were eluted with another buffer (10mM PBS, 0.15M NaCl, 20mM N-acetyl-D-glucosamine, pH 7.4). The
 20 eluted glycoproteins were dialyzed against DEAE-cellulose binding buffer (5 mM PBS, pH 8.0).

The glycoprotein fraction was then applied to the DEAE-cellulose column and washed with binding buffer
 25 until the A_{280} was less than 0.01. The binding protein was eluted with the same binding buffer with different pH values (from pH 7.4 to pH 3.0). Human recombinant ZP3 was eluted out between pH 4 to pH 6. This fraction was subsequently dialyzed against Ni-
 30 NTA (nitrilotriacetic acid) binding buffer (50mM PBS, 300mM NaCl, pH 8.0) overnight at 4°C. Before binding, the Ni-NTA resin (Qiagen, Valencia, CA) was equilibrated with ten bed volumes of Ni-NTA binding buffer. The dialyzed WGA-DEAE isolated glycoprotein
 35 was then applied to the Ni-NTA column at a flow rate of 3 to 4 resin volumes per hour. Afterwards, ten resin volumes of washing buffer (50mM PBS, 300mM NaCl, 0.1% Tween 20, 10mM 2-mercaptoethanol, pH 8.0) were used to remove non-specific binding proteins.

5 The rhZP3 was eluted using a binding buffer containing various concentrations of imidazole (Sigma).

10 *Western blotting*—The isolated glycoprotein samples were separated with SDS-PAGE and transferred to a nitrocellulose membrane by electrophoresis. A rabbit polyclonal ZP3 peptide antiserum (at 2,000 x dilution) produced by Dr. K. Hinsch and collaborators and kindly donated to us was used for immunologic identification of the rhZP3 (17). A synthetic ZP3 decapeptide (D-V-T-V-G-P-L-I-F-L) was used as antigen; this peptide antiserum detects ZP3 on the zona pellucida of human oocytes obtained for *in vitro* fertilization therapy and also on fixed ovarian tissue (18). A secondary antibody system (goat anti-rabbit IgG-Horseradish Peroxidase Antibody; Amersham Life Science, Buckinghamshire, England) was used for further identification.

25 *Semen samples and sperm capacitation*—Semen was collected by masturbation by healthy, fertile men (donors). Sperm motion parameters (% progressive motility, curvilinear and straight-line velocities, amplitude of lateral head displacement and linearity) were assessed with a computer analyzer (HTM-IVOS; Hamilton-Thorn Research, Danvers, NA). Sperm morphology was evaluated with strict criteria. The lower limits of normal parameters of samples used in the experiments were as follows: sperm concentration: $50 \times 10^6/\text{ml}$, progressive motility: 50%, and strict sperm morphology: 14% (10,11). After liquefaction, samples were washed twice in Ham's F-10 (Gibco Lab., Grand Island, NY) supplemented with 0.5% human serum albumin (HSA;

5 Irvine Sci., Santa Ana, CA). The final undisturbed
pellet was gently overlaid with 1 ml of the
culture medium and the specimen was incubated for 1
hour at 37°C in 5% CO₂ in humidified air to achieve
10 separation of the highly motile sperm fractions by
swim-up. Thereafter, aliquots were incubated under
capacitating conditions (in Ham's F-10 plus 0.5% HSA
at 37°C in 5% CO₂ in humidified air) for various
periods of time according to the experimental
design.

15

Measurement of sperm-zona pellucida binding—Salt-
stored, immature (prophase I) human oocytes were
used in the experiments. Oocytes were desalted and
microbisected into matching halves (hemizonae) using
20 a micromanipulator (Narishige, Tokyo, Japan)
following techniques published elsewhere (10,11,19).
Control and test sperm droplets (100 µl each of a
final dilution of 0.5×10^6 motile sperm/ml 1 hour
post-swim-up) were incubated separately under heavy
25 mineral oil (Sigma) with a hemizona from the same
matching pair for 4 hours at 37°C in 5% CO₂ in
humidified air. After the co-incubation period, the
hemizonae were washed to remove loosely attached
sperm using a finely drawn glass pipette, and the
30 sperm tightly bound to the outer zona surface were
counted under phase microscopy (x 200). The hemizona
assay index (HZI) was calculated as follows: # of
sperm bound for test sample / # of sperm bound for
control sample x 100. An HZI of 100 indicates no
35 inhibition whereas an HZI of 0 reflects complete
inhibition of binding.

5 Determination of the acrosome reaction—The
 proportion of live acrosome-reacted spermatozoa
 incubated under capacitating conditions was
 determined with the fluorescent probe fluorescein
 isothiocyanate-labeled *Pisum Sativum Agglutinin*
 10 (FITC-PSA, Sigma) after staining with a supravital
 stain (Hoechst 33258, Sigma) following established
 techniques (12,13). At least 200 sperm per sample
 were evaluated in duplicate at 600 x magnification
 using an epifluorescence microscope equipped with
 15 phase-contrast optics (Optiphot; Nikon, Melville,
 NY). Slides were coded and read in a blind fashion.
 Acrosome reaction was diagnosed when a total loss of
 the acrosomal cap was observed (bar pattern) or no
 immunofluorescence was seen at all (13).

20 A calcium ionophore agent (A23187, Sigma) tested at
 5 μ M and human acid solubilized zona pellucida
 tested at a final concentration of 4 zona/10 μ l were
 used as positive controls (20). In further
 25 experiments, the acrosomal status was assessed by
 transmission electron microscopy. Spermatozoa were
 fixed by mixing sperm suspensions with equal volumes
 of 2% (v/v) glutaraldehyde (in 3 mM CaCl_2 and 0.1 M
 sodium cacodylate); thereafter, samples were
 30 dehydrated twice and prepared for thin sectioning
 using previously published procedures.
 Ultrastructural examination was performed with a
 transmission electron microscope (Jeol JEM-1200 EX
 II, Peabody, MA).

35

Experimental Design

Culture medium from non-transfected (NT) PA-1 cells
 grown under similar conditions and treated following

5 the same purification procedures was used as a
negative control for sperm-zona pellucida binding
and acrosome reaction assays. The total protein
concentration of the medium was adjusted to match
the protein content of the transfected PA-1 cells
10 containing the purified rhZP3 at each experiment.

*Experiment 1: Characterization of the sperm ligand
activity of rhZP3 in competitive HZA studies*—The
ability of the rhZP3 to competitively inhibit sperm-
15 zona pellucida binding was assessed in dose-
dependency studies using the HZA. Swim up sperm were
incubated with rhZP3 (test, at a final concentration
of 0, 10, 30, 100, 250, 500 or 2,000 ng/ml of
protein) or in culture medium (control) for 30 min
20 under identical conditions. After 30 min, a hemizona
was added to the test sperm droplet whereas the
matching hemizona from the same pair was added to
the control sperm droplet. Three pairs of matching
hemizonae were tested per rhZP3 concentration per
25 semen sample in a total of three ejaculates from
different donors. These studies assessed the ability
of the rhZP3 to competitively inhibit sperm-zona
pellucida binding.

30 Additional competitive HZAs were performed where the
test sperm droplet (rhZP3) was assessed against
control sperm droplets of culture medium from non-
transfected PA-1 cells (NT). Also, competitive HZAs
were carried out where the test sperm droplet
35 (culture medium from non-transfected PA-1 cells, NT)
was assayed against sperm culture medium. Three
pairs of matching hemizonae were tested per dose per
experiment in a total of three different ejaculates.

5 These studies were carried out in order to examine the specificity of the effect of the rhZP3.

Experiment 2: Characterization of the acrosome reaction inducing activity of the rhZP3 in dose- and sperm capacitation- dependency studies—Motile sperm fractions from 29 ejaculates of 11 different donors were incubated under capacitating conditions for 3 hours and assayed for acrosome reaction using FITC-PSA. Following swim-up, sperm aliquots were pre-
10 incubated for 30 minutes with rhZP3 (30 ng/ml), A23187 (positive control), culture medium from non-transfected PA- cells (NT) or sperm culture medium (negative controls). This was the initially selected dose of rhZP3 to be tested as it had proven to
15 effect significant inhibition of sperm-zona binding in the HZA.
20

The dose-dependent effect of rhZP3 on acrosome reaction was examined in the swim-up fractions obtained from four different donors. The fractions were capacitated for 3 hours and exposed for 30 min to rhZP3 at final concentrations of 0, 7.5, 15, 30, 60, 120, 240, 480, 960 and 1920 ng/ml. The capacitation-dependency of the acrosome reaction-inducing activity of rhZP3 was assessed in the swim-up fractions of four ejaculates from four different donors. Capacitation times included: 0 (immediately post-swim-up), 1, 4, 8, 12 and 24 hours. After the capacitation period, rhZP3 was added to the sperm
25 suspension at a final concentration of 30 ng/ml.
30
35

In another set of experiments, three different ejaculates were used to compare the effects of the rhZP3 (tested at 500 ng/ml) with those of

5 solubilized zonae pellucidae and the calcium
ionophore. Further, the induction of the acrosome
reaction was assessed in parallel with FITC-PSA and
transmission electron microscopy. The goal of these
studies was to compare the morphological features of
10 the acrosome reaction between agonists and also
between the agonist-induced and basal exocytosis
rates.

*Experiment 3: Acrosome reaction- inducing activity
of rhZP3: effect on G_i proteins*-Motile sperm
fractions were obtained from the ejaculates of three
donors and incubated under capacitating conditions.
In each experiment, the test aliquot was pre-
incubated with pertussis toxin (100 ng/ml final
20 concentration) for 4 hours followed by incubation
with rhZP3 (500 ng/ml). A control aliquot was
incubated in culture medium alone for 4 hours and
then treated with rhZP3 at the same dose. After 30
min exposure to rhZP3 or control conditions, sperm
25 were assayed for acrosome reaction using FITC-PSA.

RESULTS

In vitro expression and purification of rhZP3- The
30 *in vitro* transcription and translation system
(reticulocyte lysate) demonstrated the expression of
a 47 kDa protein, the exact molecular weight as
predicted from the DNA sequence of human ZP3 (Fig.
1). The purified glycoprotein from the culture
35 medium of the transfected PA-1 cells was identified
through SDS-PAGE and Western blotting; analysis
revealed that the rhZP3 had an identical molecular
weight (approximately 65 kDa) when compared to

5 native human ZP3 from solubilized human zona
pellucida (Fig. 2) (21,22).

***Characterization of the biological activities of
rhZP3***

10

*Experiment 1: Inhibition of sperm-zona pellucida
binding by rhZP3-* Recombinant hZP3 demonstrated a
significant and dose-dependent capacity to
competitively inhibit binding under HZA conditions
15 (overall effect by analysis of variance -ANOVA-, $p < 0.0001$) (Figure 3; data in this and following
figures and tables are presented as mean \pm standard
error of the mean). The minimally effective dose was
30 ng/ml; highest inhibition of binding
20 (approximately 70%) was observed in the range of
500-2,000 ng/ml.

Studies addressing the specificity of the ligand
activity of rhZP3 are shown in Table 3. At both 30
25 ng/ml and 500 ng/ml, rhZP3 produced a significant
inhibition when tested against sperm culture medium
or against culture medium from non-transfected PA-
cells (paired t-test of rhZP3 versus control
conditions, $p < 0.01$). There were no differences
30 between the two control conditions (sperm culture
medium versus culture medium of non-transfected PA-1
cells). These results demonstrated that the effect
of rhZP3 was not only dose-dependent within the
nanomolar range but was also specific. Culture
35 medium from non-transfected PA-1 cells (cultured
under identical conditions and subjected to the same
procedures of isolation and purification as the
transfected PA-1 cells) did not produce inhibition
of binding.

5

Table 3. Specificity of the inhibitory effect of rhZP3 under HZA conditions. Recombinant human ZP3 demonstrated a significant inhibitory effect on the HZI when compared to sperm culture medium alone (Ham's - HSA) and to culture medium of non-transfected PA-1 cells (NT).

Test Reagent	versus Control	HZI (Hemizona index)
30 ng/mL rhZP3	Ham's F-10/0.5% HSA ^a	43.6 ± 3.3
30 ng/mL rhZP3	30 ng/mL NT ^a	44.5 ± 3.6
30 ng/mL NT	Ham's F-10-0.5% HSA ^b	94.2 ± 0.3
500 ng/mL rhZP3	Ham's F-10-0.5% HSA ^a	38.0 ± 2.7
500 ng/mL rhZP3	500 ng/mL NT ^a	41.8 ± 1.9
500 ng/mL NT	Ham's F-10-0.5% HSA ^b	93.4 ± 1.0

^a p<0.01 (paired t-test for test vs. control)

^b not significant

Sperm motion parameters were not significantly different under control or treatment conditions for all of the above-mentioned experiments (data not shown). This further demonstrated that the sperm-zona binding inhibition produced by rhZP3 was not due to decreased motility parameters, given additional support to the specificity of its effect.

5 *Experiment 2: Induction of acrosome reaction by*
rhZP3—Recombinant hZP3 was an effective inducer of
the acrosome reaction at 30 ng/ml (the minimally
effective dose for sperm-zona binding inhibition in
the HZA) when compared to control conditions (sperm
10 culture medium or culture medium from non-
transfected PA-1 cells) ($19 \pm 4.1\%$ live, acrosome
reacted sperm versus $9.2 \pm 3.8\%$ and $10.2 \pm 2.7\%$ live,
acrosome reacted sperm, respectively). The magnitude
of the induction of acrosome reaction was similar to
15 that of the calcium ionophore A23187 ($19.4 \pm 4.1\%$)
(overall effect by ANOVA $p < 0.0001$, with Bonferroni
correction for multiple comparisons demonstrating
differences between control conditions and rhZP3,
 $p < 0.01$ and between control conditions and A23187,
20 $p < 0.01$).

Figure 4 shows the dose-dependent agonistic effect
of rhZP3 on the induction of the acrosome reaction
(overall effect by ANOVA, $p < 0.0001$). The minimally
25 effective dose was 30 ng/ml; highest stimulation
(approximately 210% increase from baseline
conditions) was observed in the range of 500–2,000
ng/ml. There was also a significant sperm
capacitation-dependency of the acrosome reaction-
30 inducing activity of rhZP3 (ANOVA, $p < 0.03$) with
maximal stimulation observed between 8–10 hours
capacitation (data not shown).

In a different set of experiments, rhZP3 (at 500
35 ng/ml) produced a similar induction of the acrosome
reaction ($28.2 \pm 5.6\%$) when compared to solubilized
zona pellucida ($23.3 \pm 6.2\%$) and A23187 ($34.7 \pm 5.2\%$),
all of them significantly higher ($p < 0.05$) than

5 negative control (sperm culture medium, $5.7 \pm 2.8\%$).
 The ultrastructural features of the acrosome
 reaction observed by transmission electron
 microscopy were similar when comparing the effect of
 the calcium ionophore A23187 and rhZP3. Typical
 10 features of a true acrosome reaction (i.e., broken
 or absent plasma and outer acrosomal membranes with
 various degrees of loss of acrosomal content and
 exposure of the inner acrosomal membrane up to the
 equatorial region) were observed with both
 15 treatments (not shown).

Experiment 3: Effect of rhZP3 on G_i proteins- Pre-
 incubation of the motile sperm fractions with
 pertussis toxin (100 ng/ml) inhibited the induction
 20 of the acrosome reaction by rhZP3 (Figure 5).
 Incubation with the toxin, however, did not modify
 the basal rate of spontaneous acrosomal exocytosis.
 These results demonstrate that the induction of
 acrosomal exocytosis by rhZP3 is mediated via a
 25 transmembrane signaling cascade involving activation
 of pertussis toxin-sensitive G_i proteins.

Sperm motion parameters were not significantly
 different under control (sperm culture medium or
 30 culture medium of non-transfected PA-1 cells) or
 treatment (rhZP3, A23187 or pertussis toxin)
 conditions for experiments 2 and 3 (data not shown).

COMMENTS

35 Here, we successfully cloned and expressed human ZP3
 in homologous ovarian cells (PA-1) and affinity-
 purified a glycosylated product that demonstrated
 full biological activity. The rhZP3 expressed in PA-

1 cells had an estimated molecular weight of
approximately 65 kDa, within the published range of
native human ZP3 (21,22). Furthermore, the molecular
weight of the product of an *in vitro* transcription
and translation system (reticulocyte lysate) using
our recombinant vector was 47 kDa, the exact weight
of the protein backbone as predicted from the DNA
sequence (16). The results of our studies revealed
an approximately 18 kDa difference between the rhZP3
produced by the PA-1 cells and the ZP3 protein
backbone; this difference is probably due to the
presence of carbohydrate side chains. Consequently,
the affinity-purified rhZP3 expressed in PA-cells
appears to be heavily glycosylated. A biologically
active recombinant human ZP3 should present two main
properties: (i) it should demonstrate specific
ligand activity to capacitated spermatozoa; and (ii)
it should trigger acrosomal exocytosis.

In the first experiments, our rhZP3 demonstrated
ligand activity by competitively inhibiting sperm-
zona pellucida binding in the HZA. The HZA is a
useful tool to examine the mechanisms of initial
sperm-oocyte interaction by providing a homologous,
internally controlled test that assesses the
specific, irreversible and tight binding of sperm to
the zona pellucida as well as the zona-induced
acrosome reaction (10-13,19). To the best of our
knowledge, this is the first time that a rhZP3 has
been proven to competitively inhibit sperm-zona
pellucida binding in a controlled fashion and
depicting a dose-dependent inhibition under sperm
capacitating conditions. Maximal inhibition was
observed in the range of 500-2000 ng/ml. This
observation is consistent with the report of Franken

5 et al. (20) who demonstrated a similar linearity of decrease of sperm-zona pellucida binding using solubilized human zona pellucida.

10 Glycosylation appears mandatory for ZP3-ligand function (1-3). In the mouse, ZP3-ligand activity seems to reside principally in its O-linked oligosaccharides (1-3). Evidence that the amino sugar N-acetylglucosamine is the key terminal monosaccharide involved in murine gamete interaction
15 has also been presented (2). In the human, we demonstrated the involvement of fucosylated and sialylated complex-type glycans in sperm-zona pellucida binding (reviewed in 23,24). More recently, through the application of zona-lectin
20 binding and chemical- enzymatic treatment studies, direct evidence was provided for the involvement of specific carbohydrate sequences (terminal sialic acid and other fucosylated structures) on human gamete interaction (24). Since the PA-1 cell
25 glycoprotein can now be produced in large amounts, we remain hopeful that advanced methods of carbohydrate analysis will allow us to identify the saccharide epitopes responsible for sperm-zona pellucida binding in the human. In recent elegant
30 studies, it was shown that oligosaccharides located in specific serine residues in a defined locus near the carboxyl terminus encoded by exon 7 of the mouse ZP3 gene are responsible for binding in this species (5).

35 Our rhZP3 also demonstrated a potent, fast dose- and capacitation-dependent ability to induce the acrosome reaction in live spermatozoa with maximal effects also observed in the range of 500 to 2,000

5 ng/ml. The level of induction was similar to the one
observed for two well-known agonists used as
positive controls; i.e., a calcium ionophore and
solubilized human zona pellucida. The structural
features of acrosome-reacted spermatozoa showed
10 similarity to the acrosomal exocytotic changes
observed with the control agonists. Transmission
electron microscopy is still considered the "golden
standard" for the assessment of true acrosome
reactions and it was an important step to verify the
15 PSA-FITC results (25).

The PA-1 cell product, therefore, was significantly
more potent than rhZP3 produced in CHO cells (6-8).
The CHO cell product induced acrosome reaction
20 levels up to 30%, but only after 24 hours of
incubation of the sperm with the purified rhZP3 (8).
Moreover, the dose of the rhZP3 used in those
experiments was 15-20 µg/ml, whereas the PA-1 cell-
derived rhZP3 exhibited highest activity at 0.5-2
25 µg/ml, at least 10 times more potent. When an in
vitro transcription and translation system was used
to produce immobilized rhZP3 on agarose beads, the
percentage of acrosome reaction ranged from 7 to 53%
after 3 to 18 hours of sperm-beads incubation (6,7).
30 Therefore, in terms of acrosome reaction-inducing
activity, our rhZP3 appears to be more potent than
the CHO cell product and comparable to the non-
glycosylated product of an in vitro transcription
and translation system (6-8).

35 We further investigated whether rhZP3 triggered
acrosome reaction through a signaling cascade
involving heterotrimeric G proteins (3). Pertussis
toxin can cross the plasma membrane and functionally

5 inactivate G_i by ADP-ribosylating its α subunit.
Such an effect has been demonstrated using human
solubilized zona pellucida (20). Here, the rhZP3-
acrosome reaction inducing activity was inhibited by
pre-incubation of the sperm with pertussis toxin.
10 Such treatment did not affect the spontaneous rate
of acrosomal exocytosis.

The results of our studies provide strong support
for a physiological mechanism underlying the
15 functional properties of the PA-1 cell glycoprotein
product. The affinity-purified, biologically active
rhZP3 expressed in the PA-1 cells represents a
valuable tool to approach the study of human
fertilization and the design of new diagnostic and
20 contraceptive strategies.

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